Transport of acetate in mutants of Saccharomyces cerevisiae defective in monocarboxylate permeases

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Abstract

The strain Saccharomyces cerevisiae W303-1a, able to grow in a medium containing acetic acid as the sole carbon and energy source, was subjected to mutagenesis in order to obtain mutants deficient in monocarboxylate permeases. Two mutant clones exhibiting growth in ethanol, but unable to grow in a medium with acetic acid as the sole carbon and energy source, were isolated (mutants Ace12 and Ace8). In both mutants, the activity for the acetate carrier was strongly affected. The mutant Ace8 revealed not to be affected in the transport of lactate, while the mutant Ace12 did not display activity for that carrier. These results reinforced those previously found in the strain IGC 4072, where two distinct transport systems for monocarboxylates have been described, depending on the growth carbon source. It is tempting to postulate that the Ace8 mutant seems to be affected in the gene coding for an acetate permease. In contrast, the absence of activity for both monocarboxylate permeases in mutant Ace12 could be attributed to a mutation in a gene coding for a regulatory protein not detected before. z 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Saccharomyces cerevisiae; Yeast; Transport; Monocarboxylate

1. Introduction

The transport of monocarboxylic acids through the plasma membrane, the first step of their metabolism, has been elucidated in the strain Saccharomyces cerevisiae IGC 4072 [1,2]. Two distinct monocarboxylate carriers were found, regarding either their specificity or regulation. An acetate-propionate-formate permease is present in acetic acid-, lactic acid- or ethanol-grown cells, while in lactic acid-grown cells, another permease, shared by lactate-pyruvate-propionate-acetate, is also inducible. These transporters are subjected to glucose repression, therefore, no measurable activity was found in glucose-grown cells.

In S. cerevisiae it is well established that the intracellular metabolism of acetic acid or ethanol, implies the operationality of the anaplerotic glyoxylate cycle and the gluconeogenesis pathways [3]. Regarding the utilisation of ethanol, two additional steps are required. First, the oxidation to acetaldehyde catalysed by alcohol dehydrogenase II and after, a second oxidation to acetic acid catalysed by aldehyde dehydrogenase [3]. Our work focused on the obtention of mutants unable to utilise acetic acid, but not a¡ected in the capacity to grow with ethanol as the sole carbon and energy source. With this strategy, mutants

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specifically affected on monocarboxylate permease(s) activity were found. Studies regarding the physiological and genetic characterisation of such mutants are described.

2. Materials and methods

2.1. Microorganism and growth conditions

As starting material the strains S. cerevisiae W303-1a and W303-1K were used. The cultures were maintained on slants of yeast extract (0.5%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v). For growth, a medium with 1% (w/v) yeast extract and 1% (w/v) peptone (YP medium) or in Difco yeast nitrogen base, supplemented with the adequate requirements for prototrophic growth (YNB medium) was used. Carbon sources were either glucose (2%, w/v), D,L-lactic acid (0.5%, v/v, pH 5.0), sodium pyruvate (0.5%, w/v, pH 5.0), acetic acid (0.5%, v/v, pH 5.0), glycerol (1%, w/v) or ethanol (1%, w/v). Solid media were prepared adding agar (2%, w/v) to the respective liquid media. Growth was carried out at 28^30°C, both in solid and liquid media.

2.2. Repression and derepression conditions for transport assays

For growth of yeast cells under repression conditions, the medium YP with glucose (2%, w/v) was used. Derepressed conditions were obtained by incubating cells, previously grown under repression conditions, in YNB medium with acetic acid or D,L-lactic acid, for 6 or 4 h, respectively.

2.3. Genetic methods and techniques

Mutagenesis was carried out with UV radiation as previously described [4]. After mutagenesis, cells were plated on YP medium with ethanol (1%, w/v) and replica-plated on YNB with acetic acid (0.5%, w/v, pH 5.0). Crosses, isolation of diploids, sporulation and tetrad analysis were done by standard methods [5].

2.4. Transport assays

Exponential growing cells, or cells incubated under derepressed conditions, were harvested by centrifugation, washed twice in ice-cold deionised water and resuspended in ice-cold deionised water to a final concentration of about 25^45 mg dry wt. ml^{-1}. The initial uptake rates of labelled monocarboxylic acids were estimated using 10-ml conical centrifuge tubes containing 30 μl of 0.1 M potassium phosphate buffer at the desired pH and 10 μl of the yeast suspension. After 4 min of incubation at 25°C in a water bath, the reaction was started by the addition of 10 μl of an aqueous solution of labelled acid (3000^3300 dpm $nmol^{-1}$) at the desired concentration and pH. The reaction was stopped by dilution with 5 ml of ice-cold water. Sampling times were 0, 5 and 10 s, over which the uptakes of labelled acids were linear. The reaction mixtures were filtered immediately through Whatman GF/C membranes, the filters washed with 10-ml of ice-cold water and transferred to the scintillation fluid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer, with dpm correction. The effect of non-labelled substrates on the initial uptake velocities of labelled acid was assayed by adding simultaneously the labelled and non-labelled substrates (carboxylic acids or ethanol). The radioactively labelled substrates utilised were: D,L-[1-^{14}C]lactic acid, sodium salt (Amersham) and [U-^{14}C]acetic acid, sodium salt (Amersham). Results were corrected for non-specific ^{14}C adsorption to the filters and/or the cells, determined by diluting the cells with 5 ml ice-cold distilled water, before the addition of labelled carboxylic acid. The values estimated represented less than 10% of the total incorporated radioactivity. To determine the best fitting transport kinetics to the experimental initial uptake rates values and to estimate the kinetic parameters, a computer-assisted non-linear regression analysis (GraphPAD software, San Diego CA, USA) was used. All the experiments were repeated at least three times and the data reported here represent the average values.

2.5. Determination of enzyme activities

Acetyl-CoA synthetase, phosphoenolpyruvate carboxykinase, malate dehydrogenase and isocitrate lyase were assayed by conventional spectrophotometric procedures [6^8].

3. Results and discussion

3.1. Searching for respiratory competent mutants unable to grow on acetic acid

Two mutant clones (Ace8 and Ace12), derived from the strain of S. cerevisiae W303-1a, defective in acetic acid utilisation were isolated from two independent mutagenic experiments, where about 20 000 cells were screened. For both mutants, no growth was observed either in liquid or on solid YNB-acetic acid medium. The phenotype of the mutants was evaluated regarding their ability to grow in other carbon sources, such as ethanol, glycerol, glucose and D,L-lactic acid. All these substrates were used by the mutant Ace8 as the only carbon and energy sources. Furthermore, the values estimated for specific growth rates, as well as for final OD (640 nm) of the cultures, were of the same order of magnitude of those found in the wild-type strain (not shown). A similar growth pattern was observed for the mutant Ace12, except in D,L-lactic acid medium where no growth was found. Representative results of these studies are shown in Fig. 1. In this figure, the wild-type strain (W303-1a) and the strain CJM197, a gluconeogenic mutant [9], were included as a positive and a negative control, respectively, regarding their ability to use ethanol, lactic acid and acetic acid. As can be seen, the two mutant clones could be distinguished by their phenotypes. Although a slight growth for the strain CJM197 could be observed in YP solid medium with ethanol, when the same experiments were carried in YNB liquid medium with ethanol, no growth occurred. It should be noted that YNB solid medium with ethanol could not be used since the wild-type strain was unable to grow on this medium.

In order to elucidate the intracellular metabolic functionality of these mutants, the specific activity of representative enzymes of the main metabolic routes was determined, namely phosphoenolpyruvate carboxykinase, isocitrate lyase, malate dehydrogenase and acetyl-CoA synthetase. Activity for all these enzymes was found, in spite of some variation on the values determined in the distinct strains (Table 1).

3.2. Genetic analysis

The diploid strains derived from the genetic crosses either of Ace8 or Ace12 with the wild-type

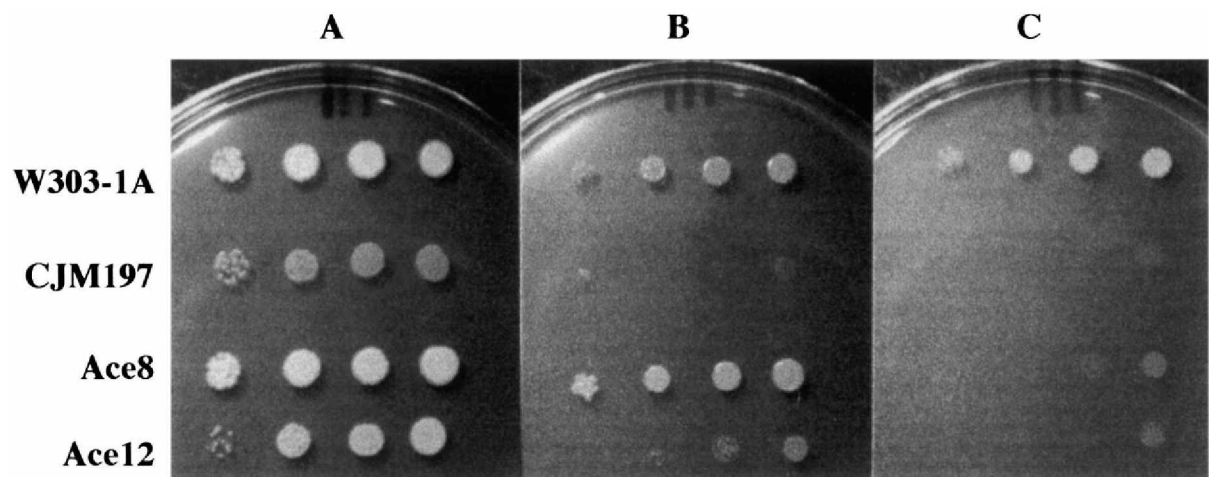

Fig. 1. Growth phenotypes of S. cerevisiae W303-1a, CJM197, Ace8 and Ace12 in the following solid media: (A) YP-ethanol 2% (w/v); (B) YNB-lactic acid (0.5%, w/v, pH 5.0); and (C) YNB-acetic acid (0.5%, w/v, pH 5.0). The strain CJM197, included in this figure, is a mutant disrupted on the gene coding the enzyme fructose-1,6-bisphosphatase [9].

Table 1
Specific activity of the enzymes phosphoenolpyruvate carboxykinase (PCK), isocitrate lyase (ICL), malate dehydrogenase (MD) and acetyl-CoA synthetase (ACS)

Strain	Specific activity (mU mg protein^{-1}) PCK			
		ICL	MD	ACS
W303-1a	110	30	87	6
Ace8	88	32	90	10
Ace12	51	22	99	13

Enzyme activities were measured in cell extracts from overnight cultures of S. cerevisiae grown in YP plus glycerol and ethanol. Enzyme assays were performed in triplicate.

strain W303-1K, displayed the same phenotype found on the wild-type parental cells, regarding the ability to utilise all the carbon sources discussed in the previous section. In 10 complete tetrads, originated from the parental cross Ace8UW303-1K, growth on acetic acid medium segregated 2 acetate‡ :2 acetate3 and no distinct phenotypes were found in the remaining carbon sources. The analysis processed in 10 complete tetrads derived from the parental cross Ace12UW303-1K, gave the same progeny segregation pattern. However, those descendant cells that failed to grow in acetic acid did not grow in lactic acid-medium as well. The diploid strain deriving from the genetic cross between a clone F1-Ace8 (acetate3) and a clone F1-Ace12 (acetate3) behaved as the wild-type strain, being able to use acetic and lactic acids, as the only carbon and energy sources and exhibiting activity for both monocarboxylate permeases. These results pointed to the presence of a monogenic recessive nuclear mutation in each mutant clone, belonging to distinct complementation groups.

3.3. Acetate transport

The uptake of labelled acetic acid, at pH 5.0, was measured in cells of the W303-1a derepressed in YNB-acetic acid medium (Fig. 2). The application of a computer-assisted non-linear regression analysis to the experimental data (acid concentration 0.04^2.0 mM), agreed with the presence of a mediated transport system and the values estimated for K_m and V_{max} were, respectively, 0.56þ0.19 mM and 1.36þ0.18 nmol s^{-1} mg dry wt.$^{-1}$. The kinetics for the uptake of labelled acetic acid at pH 5.0 in acetic

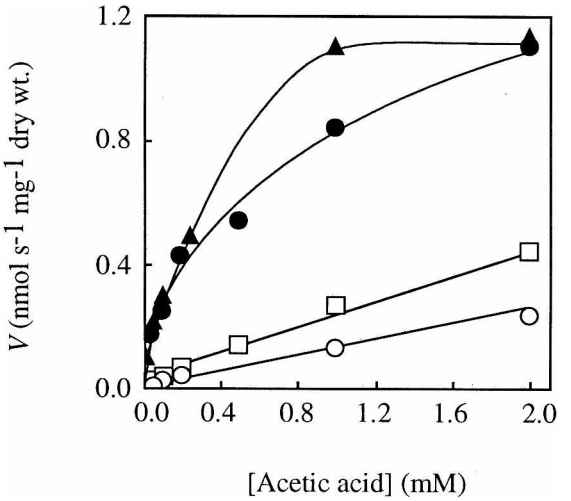

Fig. 2. Initial uptake rates of labelled acetic acid, pH 5.0, by YNB acetic acid-derepressed cells of S. cerevisiae strains W303-1a (b), mutant Ace8 (E) and mutant Ace12 (a), as a function of the acid concentration. The velocities for labelled acetic acid previously reported by Casal et al. [2] found in acetic acid-grown cells of the strain IGC 4072 are also represented (R).

acid-grown cells of the strain IGC 4072, previously published [2], is also shown in Fig. 2. When the mutant strains were analysed for their capacity to transport labelled acetic acid, a di¡erent behaviour was found (Fig. 2). For the mutant Ace12, the uptake conformed to a first order kinetics, indicating simple di¡usion of the acid. From these results, a value of 0.34þ0.02 Wl s^{-1} mg dry wt.$^{-1}$ was estimated for the di¡usion constant (K_d) of the undissociated acid. Regarding the mutant Ace8, the most probable kinetics fitted to a transport mechanism with two components, a simple di¡usion for the undissociated acid and residual activity for a carrier, the following parameters were estimated: K_m, 0.06þ0.08 mM; V_{max}, 0.04þ0.02 nmol s^{-1} mg dry wt.$^{-1}$; and K_d, 0.61þ0.03 Wl s^{-1} mg dry wt.$^{-1}$. In the wild-type strain, the acetate permease was inhibited by the presence of propionic acid (Fig. 3A). The inhibition was competitive indicating that both acids shared the same carrier as reported for the strain IGC 4072 of S. cerevisiae. Furthermore, the presence of ethanol also inhibited the acetate transport, but in a non-competitive way [10], reinforcing that a permease was present (Fig. 3A). The same methodology carried out in cells of the mutant Ace8 showed a

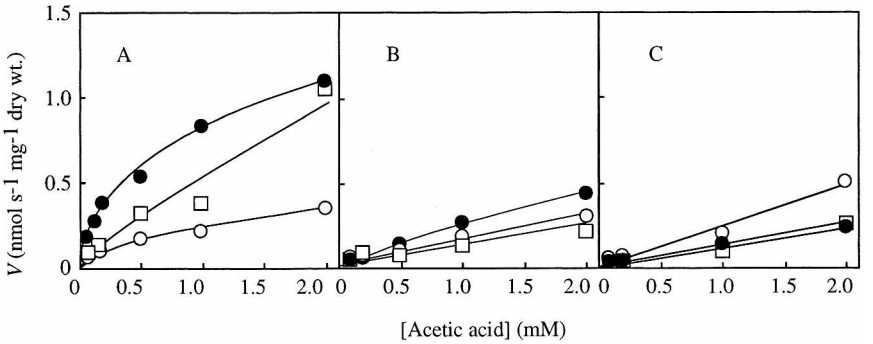

Fig. 3. Initial uptake rates of labelled acetic acid by YNB acetic acid-derepressed cells of S. cerevisiae W303-1a (A), mutant Ace8 (B) and mutant Ace12 (C), as a function of the acid concentration. b, Absence of other non-labelled substrate; E, presence of 2.5 mM of propionic acid; a, presence of 2.7 mM of ethanol.

slightly inhibitory e¡ect by ethanol and propionic acid in the initial velocities of the acid transport (Fig. 3B), probably due to a residual activity of the acetate carrier. In cells of the mutant Ace12, propionic acid had no e¡ect on the acetate transport, while ethanol induced a stimulatory e¡ect (Fig. 3C). This, in all likelihood, is the behaviour found on glucose repressed cells, where no measurable activity for the acetate permease is found [2], therefore indicating the absence of an operational carrier for acetate in Ace12 mutant.

3.4. Lactate transport

As it has previously been reported, in lactic acid grown-cells of S. cerevisiae IGC 4072, that besides the above-described acetate-propionate-formate carrier [2], another permease, shared by lactate-pyruvate-propionate-acetate, inducible by D,L-lactic acid, is present [1]. Concerning the kinetics of the transport of labelled lactic acid in lactic acid-derepressed cells of the strain W303-1a, the application of a computer-assisted non-linear regression analysis to the experimental data (acid concentration 0.02^ 2.0 mM), agreed with the presence of a mediated transport system, without contamination of simple di¡usion component of the undissociated acid, with the following parameters at pH 5.0: K_m, 0.44 þ 0.13 mM; and V_{max}, 0.22 þ 0.02 nmol s^{31} mg dry wt.^{31} (Fig. 4). In lactic acid-derepressed cells of Ace8 mutant, the transport of labelled lactic acid followed a Michaelis^Menten kinetics, with the K_m, 0.62 þ 0.13 mM and the V_{max}, 0.15 þ 0.01 nmol s^{31} mg dry wt.^{31} (Fig. 4). In the mutant Ace12, no measurable activity for the lactate carrier was found (Fig. 4) and the best fitting was in conformity with a simple di¡usion of

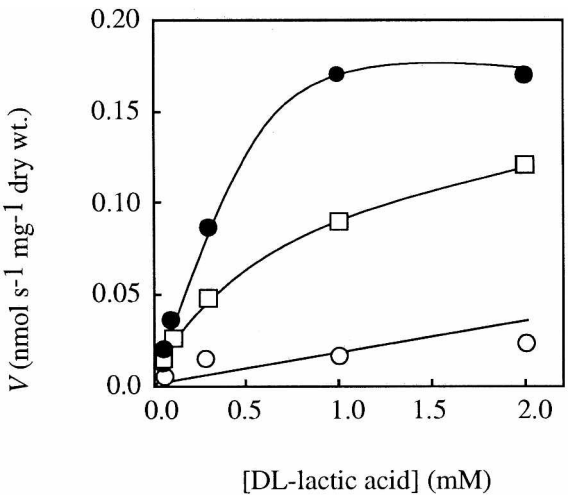

Fig. 4. Initial uptake rates of labelled lactic acid by YNB lactic acid-derepressed cells of S. cerevisiae strains W303-1a (b), mutant Ace8 (E) and mutant Ace12 (a), as a function of the acid concentration.

the undissociated acid with the value of 0.21 þ 0.02 Wl s^{-1} mg dry wt.$^{-1}$ for K_d.

In summary, from the results obtained, it is reasonable to postulate that mutant Ace8 carries a mutation in the gene coding for the acetate permease and the mutant Ace12 is probably a¡ected in a gene involved in the control of the expression/activity of the monocarboxylate permeases, the existence of which has not previously been detected. Therefore, the results reinforced previous evidence indicating the presence of a family for monocarboxylate permeases, with distinct substrate specificities and mechanisms of regulation in S. cerevisiae.

Acknowledgments

This study was supported by a Portuguese research programme (contract PRAXIS/2/2.1/BIO/1068/95). S.P. received a fellowship from PRAXIS XXI-BM4229.

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